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(54) Title: A NEW METHOD FOR THE DIAGNOSIS AND PROGNOSIS OF MALIGNANT DISEASES

(57) Abstract: Methods for the diagnosis of cancer by determining the neoplastic status of a cell by probing the cell plasma membrane for the presence of nucleolin are provided, as are kits to carry out such tests.

METHOD FOR THE DIAGNOSIS AND PROGNOSIS OF MALIGNANT DISEASES

The invention is based on the discovery of a correlation between nucleolin cellular plasma membrane expression and the presence and aggressiveness of neoplastic cells. Thus, the invention is drawn to the detection and prognosis of pre-malignant conditions and malignant diseases and disorders and kits for detection and prognosis.

BACKGROUND

Being well-prepared for battle engenders success; when the foe is cancer, early detection results in a greater likelihood that medical intervention will be successful. At early stages, treatments can often be targeted only to the affected tissues, diminishing side effects. If not caught early, cancer cells may metastasize and spread throughout the body. The prognosis in this case is more dire, and medical treatments are often applied systemically, killing not only cancer cells, but large numbers of healthy cells.

A hallmark of a cancer cell is uncontrolled proliferation. Cancer cells may also exhibit morphological and functional aberrations. Cancer cells may display less organized cellular morphology; for example, losing the asymmetric organelle and structural organization (cell polarity) that allows for proper cell function. Cell-cell and cell-substratum contacts, the specificities of which are also necessary for normal function, are often modulated or lost. Functionally, the cells may carry on few, if any, wild-type functions, or may have exaggerated, unregulated normal functions, such as hormone secretion. Such cells regress to early developmental stages, appearing less differentiated than their wild-type (*i.e.*, normal) parents.

Cancer cells also often mis-express or mis-target proteins to inappropriate cellular compartments. Proteins may be up- or down-regulated; even proteins not usually expressed by a specific cell type can be expressed by the transformed counterpart. Protein mis-expression can have a plethora of downstream cellular effects, including drastic changes in membrane composition, organelle formation, or

physiology. Mis-targeting of proteins (and other molecules, such as lipids, *etc.*) also contributes to the loss of cell polarity.

Detecting cancer growths are complicated by a variety of factors, including protein mis-expression and cellular dedifferentiation. For example, cancers can proceed gradually, being present in only small numbers that are difficult to detect. A common diagnostic approach is the use of certain proteins as "markers" to distinguish cancer cells from healthy ones. For example, prostate cancer diagnoses often use prostate-specific antigen (PSA). These assays require that a marker be defined, have detection agents, and be readily available. However, a marker that is present in a many, if not most, cancers has yet to be defined. Such a marker would remarkably facilitate diagnosis.

SUMMARY

In a first aspect, the invention is drawn to methods of determining a neoplastic state of a cell, comprising detecting the presence of nucleolin on the plasma membrane of a cell, including mammalian cells, such as human; the cell may also be lysed. Plasma membrane nucleolin is detected by using an anti-nucleolin agent, such as anti-nucleolin antibodies or oligonucleotides, including those of sequences of SEQ ID NOs:1-7; 9-17; 19-31.

In a second aspect, the invention is drawn to methods of determining a phenotype of a neoplastic cell, comprising quantifying the amount of plasma membrane nucleolin of the cell, including mammalian cells, such as human; the cell may also be lysed. Plasma membrane nucleolin is detected by using an anti-nucleolin agent, such as anti-nucleolin antibodies or oligonucleotides, including those of sequences of SEQ ID NOs:1-7; 9-17; 19-31.

In another aspect, the invention is drawn to kits for determining a neoplastic state of a cell, comprising an anti-nucleolin agent and a control sample. Such kits may also contain reagents that bind to nucleolin, reagent-nucleolin complexes and other components, such as a fixative, a buffer, plasticware, serum, serum albumin, non-fat milk, membranes and instructions.

In another aspect, the invention pertains to methods of detecting small lung cell carcinoma in a subject, comprising collecting a sample containing lung cells from

a subject ; and detecting the presence of plasma membrane nucleolin in the cells. Samples include sputum; subjects may be human.

In yet another aspect, the invention is drawn to methods of diagnosing tumor, pre-malignant or malignant cells, comprising collecting a sample from a subject comprising cells, sending the sample to a testing center, determining the neoplastic state of the cells by probing for cell plasma membrane nucleolin.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows nuclear nucleolin staining in various cell lines. Shown are immunofluorescent (B, D, F, H) and parallel phase contrast micrographs (A, C, E, G). Cell lines that were analyzed were: DU145 prostate cancer cells (A, B), MDA-MB-231 breast cancer cells (C, D), HeLa cervical cancer cells (E, F) and HS27 normal skin cells (G, H). An anti-nucleolin antibody was used; the cells were permeabilized before staining to allow the antibody access to the cytoplasmic and nuclear compartments.

FIG. 2 shows plasma membrane nucleolin staining in the cell lines shown in Figure 1. Shown are immunofluorescent (B, D, F, H) and parallel phase contrast micrographs (A, C, E, G). Cell lines that were analyzed were: DU145 prostate cancer cells (A, B), MDA-MB-231 breast cancer cells (C, D), HeLa cervical cancer cells (E, F) and HS27 normal skin cells (G, H). An anti-nucleolin antibody was used; the cells were not permeabilized before staining, allowing the antibody access to only the plasma membrane.

FIG. 3 shows the comparative proliferation rates of cell lines as measured by MTT assay. Square, DU145; diamonds, HeLa; circles, HS27. Although MDA-MB-231 was not included in this experiment, proliferation rates for these four cell lines have been determined to be DU145 > MDA-MB-231 > HeLa > HS27. Note that the cell lines with high levels of plasma membrane nucleolin correspond to those with the most rapid proliferation (DU145 and HeLa; see Figure 2).

FIG. 4 shows phase contrast (B,D) and immunofluorescent image (A,C) of a paraffin-embedded specimen resected from a patient with squamous cell carcinoma of the head and neck. The specimen was stained for plasma membrane nucleolin and counter-stained with propidium iodide to show cell nuclei. Images (C) and (D) show images (A) and (B) overlaid with markings to better show nucleolin staining. The area **1** encompassed by the white line includes intense nucleolin staining, while the areas outside of **1** show little **3** or no **2** signal.

FIG. 5 shows phase contrast (B, D) and immunofluorescent images (A, C) of small cell (NCI-H82) and non-small cell lung (NCI-H1299) cancer cell lines placed onto a microscope slide using a cytospinner. Samples were stained for plasma membrane nucleolin and counter-stained with propidium iodide to show cell nuclei. Cells with exceptionally well-stained plasma membranes are denoted by asterisks (*).

FIG. 6 shows phase contrast (B, D, F) and immunofluorescent images (A, C, E) of peripheral blood (A, B) or bone marrow (C, D and E, F) from human subjects. Samples were stained for plasma membrane nucleolin and counter-stained with propidium iodide to show cell nuclei. Highly stained cells for nucleolin are marked with an asterisk (*); these were only seen in those patients suffering from carcinomas (A,B and C,D), while cells from a healthy patient did not display any plasma membrane nucleolin staining (E, F).

DETAILED DESCRIPTION

The invention is based on the discovery of a correlation between nucleolin plasma membrane expression and the presence and aggressiveness of neoplastic cells.

The invention provides compositions and methods for the detection of neoplastic cells, such as malignant and pre-malignant cells, using a neoplastic antigen that is broadly expressed, nucleolin. However, the expression of nucleolin is insufficient to indicate malignancy (nucleolin is found in every nucleated cell); but the localization of nucleolin on the cell surface is indicative. While increased amounts of nuclear nucleolin, as visualized by silver staining, has been used as a diagnostic tool

for cancer, the unexpected discovery that nucleolin found on the cell surface correlates with a pre-malignant or malignant phenotype can facilitate cancer diagnosis and prognosis.

The advantages of using surface-localized nucleolin include:

(1) Improved accuracy. Assaying for plasma membrane nucleolin more accurately identifies the malignant phenotype compared to silver-staining for nuclear nucleolin.

(2) Specificity. Plasma membrane nucleolin is not normally observed in the plasma membrane of most wild-type (healthy) cells. Thus, unlike many other diagnostic tests that require analyzing the amount of a marker, plasma membrane nucleolin detection can be used qualitatively. A more fail-proof "yes-no" type of test is therefore feasible. This aspect also allows for testing on both single cells and tissue samples.

(3) Broad applicability. Many different cancers may be detected by examining for plasma membrane nucleolin.

(4) Multifunctional. The methods are prognostic as well as diagnostic: the rate of cellular proliferation typically correlates with levels of plasma membrane nucleolin.

While investigating the anti-proliferative activity of non-antisense guanosine-rich oligonucleotides (GROs) on cancer cells, it was found that such anti-proliferative GROs bind nucleolin to exert their effects (Bates *et al.*, 1999; Miller *et al.*, WO 00/61597, 2000). Nucleolin (Bandman *et al.*, US Patent No. 5,932,475, 1999) is an abundant, non-ribosomal protein of the nucleolus, the site of ribosomal gene transcription and packaging of pre-ribosomal RNA. This 707 amino acid phosphoprotein has a multi-domain structure consisting of a histone-like N-terminus, a central domain containing four RNA recognition motifs and a glycine/arginine-rich C-terminus and has an apparent molecular weight of 110 kD. Its multiple domain structure reflects the remarkably diverse functions of this multifaceted protein (Ginisty *et al.*, 1999; Srivastava and Pollard, 1999; Tuteja and Tuteja, 1998). Nucleolin has been implicated in many fundamental aspects of cell survival and proliferation. Most understood is the role of nucleolin in ribosome biogenesis. Other

functions may include nucleocytoplasmic transport, cytokinesis, nucleogenesis and apoptosis.

Nucleolin synthesis has been correlated with increased rates of cell division (cell proliferation); nucleolin levels are therefore higher in tumor cells compared to most normal cells (Tuteja and Tuteja, 1998). Nucleolin is one of the nuclear organizer region (NOR) proteins whose levels, as measured by silver staining, are assessed by pathologists as a marker of cell proliferation and an indicator of malignancy (Derenzini, 2000).

Also present in the cell plasma membrane in a few cell types, such as lymphocytes and inner medullary collecting duct cells, nucleolin has been hypothesized to function as a receptor (*e.g.*, Callebaut *et al.*, 1998; Sorokina and Kleinman, 1999). However, the role of plasma membrane nucleolin is not well understood. In addition, it is not clear whether the plasma membrane nucleolin is identical to the nucleolar protein, or if it represents a different isoform or nucleolin-like protein. However, the expression of plasma membrane nucleolin is specific to neoplastic cells (such as malignant or pre-malignant); thus the function of plasma membrane nucleolin need not be known for diagnostic and prognostic purposes.

Definitions

neoplasm, malignancy, tumor, cancer cells

A neoplasm is an abnormal tissue growth resulting from neoplastic cells, cells that proliferate more rapidly and uncontrollably than normal cells. Usually partially or completely structurally disorganized, neoplasms lack functional coordination with the corresponding normal tissue. Neoplasms usually form a distinct tissue mass that may be either benign (tumor) or malignant (cancer).

Cancer cells invade surrounding tissues, may metastasize to distant sites, and are likely to recur after attempted removal, causing death of a subject if not adequately treated. In addition to structural disorganization, cancer cells usually regress to more primitive or undifferentiated states (anaplasia), although morphologically and biochemically, they may still exhibit many functions of the corresponding wild-type cells. Carcinomas are cancers derived from epithelia; sarcomas are derived from connective tissues.

Cancers may be more aggressive or less aggressive. The aggressive phenotype of a cancer cell refers to the proliferation rate and the ability to form tumors and metastasize in nude mice. Aggressive cancers proliferate more quickly, more easily form tumors and metastasize than less-aggressive tumors.

neoplastic state

The term "neoplastic state" refers to three conditions: normal, pre-malignant and malignant. "Normal" refers to a growth or cell that is clinically normal (healthy). "Pre-malignant" refers to a growth or cell that is on the pathway to malignancy, but at the time of examination, would not be classified as malignant by conventional methods. "Malignant" refers to a cell or growth that has at least one of the following properties: locally invasive, destructive growth and metastasis.

GROs and other polypeptide-binding oligonucleotides

Oligonucleotides are available that specifically bind to polypeptides, such as nucleolins. Examples of such are GROs, which are guanosine-rich oligonucleotides. Characteristics of GROs include:

(1) having at least 1 GGT motif

(2) preferably having 4-100 nucleotides, although GROs having many more nucleotides are possible

(3) having chemical modifications to improve stability.

Especially useful GROs form G-quartet structures, as indicated by a reversible thermal denaturation/renaturation profile at 295 nm (Bates *et al.*, 1999). Preferred GROs also compete with a telomere oligonucleotide for binding to a target cellular protein in an electrophoretic mobility shift assay (Bates *et al.*, 1999).

Other oligonucleotides may have high binding specificity for nucleolin.

anti-nucleolin agent

An "anti-nucleolin agent" binds to nucleolin. Examples include anti-nucleolin antibodies and certain oligonucleotides.

Embodiments

The following embodiments are given as examples of various ways to practice the invention. Many different ways of practicing the invention are also possible.

In all embodiments, the underlying principle is to specifically differentiate between plasma membrane nucleolin and nuclear nucleolin. Plasma membrane nucleolin, as discovered by the applicants, correlates with cells that are in a neoplastic state; furthermore, the amount of plasma membrane nucleolin also indicates the aggressiveness of these cells; that is, the higher the plasma membrane nucleolin expression, the more aggressive the cells. Various techniques allow a user to differentiate between nuclear and plasma membrane nucleolin. Detection techniques, wherein the nucleolin-detecting reagents have exclusive access to extracellular portions of the cell (and consequently cell-plasma membrane nucleolin), or biochemical techniques, wherein either the surface plasma membrane and/or surface proteins are separated from other cellular components and compartments, are also useful.

In an embodiment, nucleolin is detected directly on the cell surface. A cell is isolated from a subject and plasma membrane nucleolin detected using an agent that binds nucleolin. Cells may be isolated by any known technique. An isolated cell may comprise a larger tissue sample containing cells that are not neoplastic. Detection procedures use anti-nucleolin antibodies that bind extracellular nucleolin epitopes; these antibodies may be directly labeled or when bound, detected indirectly. Other useful plasma membrane nucleolin detection agents include GROs that specifically bind nucleolin. Useful procedures, such as fluorescence-activated cell sorting (FACS) or immunofluorescence, employ fluorescent labels, while other cytological techniques, such as histochemical, immunohistochemical and other microscopic (electron microscopy (EM), immuno-EM) techniques use various other labels, either colorimetric or radioactive. The various reagents may be assembled into kits.

In another embodiment, cells are isolated from a subject and extracted. Plasma plasma membranes and/or proteins are then isolated (such as *via* differential extraction, or differential physical cell disruption, differential centrifugation of detergent-extracted cells, *etc.*), and then nucleolin detected in the isolated membranes using an agent that binds nucleolin. In general, useful techniques to detect nucleolin include those wherein the extract is placed on a substrate, and the substrate probed with a nucleolin-detecting reagent. Examples of such techniques include polypeptide dot blots and Western blots, biochips, protein arrays, *etc.*. Other detection formats

include enzyme-linked immunosorbent assays (ELISAs) in their manifold manifestations (Ausubel *et al.*, 1987). In embodiments wherein plasma membrane surface molecules are physically separated from most of the other cellular components and compartments, the nucleolin-binding agents need not specifically recognize any extracellular portions of nucleolin. The various reagents may be assembled into kits.

In a further embodiment, the methods of the invention are directed to detecting lung cancer, such as lung small cell carcinomas. Plasma membrane nucleolin expression is useful for detection and prognosis.

In yet another embodiment, methods of treating cells in a neoplastic state including cancer and tumor cells are provided, exploiting plasma membrane nucleolin that acts as a beacon. Administration of anti-nucleolin antibodies, which may be conjugated to a toxin or other means of stimulating cell death or incurring cell necrosis, results in the removal of plasma membrane nucleolin-expressing cells.

Practicing the invention

The methods of the invention include in part the steps of collecting cells from a subject and detecting plasma membrane nucleolin. The following, not meant to limit the invention, is presented to aid the practitioner in carrying out the invention, although other methods, techniques, cells, reagents and approaches can be used to achieve the invention.

Cells

Cells or tissue samples are collected from a subject. The subject is a vertebrate, more preferably a mammal, such as a monkey, dog, cat, rabbit, cow, pig, goat, sheep, horse, rat, mouse, guinea pig, *etc.*; and most preferably a human. Any technique to collect the desired cells may be employed, including biopsy, surgery, scrape (inner cheek, skin, *etc.*) and blood withdrawal. Any appropriate tool may be used to carry out such tasks. It is not necessary to isolate the test population (*i.e.*, those cells being tested for neoplastic state) from those cells and tissues (contaminating material) that are not being tested, except in some cases using biochemical methods that include extraction. In this last case, the test population

need not be completely isolated from contaminating materials, but should either predominate or be easily distinguishable (*e.g.*, morphologically (structurally, specific markers) or biochemically).

For those methods that analyze lung carcinomas, sputum collection is an attractive and easily obtained sample. The term "sputum" as used herein refers to expectorated matter made up of saliva and discharges from the respiratory airways. Sputum is a highly complex material that has a pronounced gel-like structure.

For collection of sputum, Byrne, *et al.*, (Byrne, 1986) suggest that the patient collect material, raised by several deep coughs, in a container with a lid. Alternatively, sputum can be collected by using a bronchoscope (Kim *et al.*, 1982). Specific devices or agents may be used to facilitate sputum collection (Babkes *et al.*, US Patent 6,325,785, 2001; King and Speert, US Patent 6,339,075, 2002; Rubin and Newhouse, US Patent 5,925,334, 1999). Other methods of sputum collection are also available.

Cell culture

In some cases, culturing the harvested cells is desirable to augment their numbers so that plasma membrane nucleolin detection is facilitated. Suitable media and conditions for generating primary cultures are well known. The selection of the media and culture conditions vary depending on cell type and may be empirically determined. For example, skeletal muscle, bone, neurons, skin, liver, and embryonic stem cells are grown in media that differs in their specific contents. Furthermore, media for one cell type may differ significantly from laboratory to laboratory and institution to institution. To keep cells dividing, serum, such as fetal calf serum (FCS) (also known as fetal bovine serum (FBS)), is added to the medium in relatively large quantities, 5%-30% by volume, depending on cell or tissue type. Other sera include newborn calf serum (NCS), bovine calf serum (BCS), adult bovine serum (ABS), horse serum (HS), human, chicken, goat, porcine, rabbit and sheep sera. Serum replacements may also be used, such as controlled process serum replacement-type (CPSR; 1 or 3) or bovine embryonic fluid. Specific purified growth factors or cocktails of multiple growth factors can also be added or sometimes substituted for

serum. Specific factors or hormones that promote proliferation or cell survival can also be used.

Examples of suitable culture media include Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium Eagle (MEM), Basal Medium Eagle (BME), Click's Medium, L-15 Medium Leibovitz, McCoy's 5A Medium, Glasgow Minimum Essential Medium (GMEM), NCTC 109 Medium, Williams' Medium E, RPMI-1640, and Medium 199. A medium specifically developed for a particular cell type/line or cell function, *e.g.* Madin-Darby Bovine Kidney Growth Medium, Madin-Darby Bovine Kidney Maintenance Medium, various hybridoma media, Endothelial Basal Medium, Fibroblast Basal Medium, Keratinocyte Basal Medium, and Melanocyte Basal Medium are also known. If desired, a protein-reduced or -free and/or serum-free medium and/or chemically defined, animal component-free medium may be used, *e.g.*, CHO, Gene Therapy Medium or QBSF Serum-free Medium (Sigma Chemical Co.; St. Louis, MO), DMEM Nutrient Mixture F-12 Ham, MCDB (105, 110, 131, 151, 153, 201 and 302), NCTC 135, Ultra DOMA PF or HL-1 (both from Biowhittaker; Walkersville, MD), may be used.

The medium can be supplemented with a variety of growth factors, cytokines, serum, *etc.*, depending on the cells being cultured. Examples of suitable growth factors include: basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), transforming growth factors (TGFα and TGFβ), platelet derived growth factors (PDGFs), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), insulin, erythropoietin (EPO), and colony stimulating factor (CSF). Examples of suitable hormone additives are estrogen, progesterone, testosterone or glucocorticoids such as dexamethasone. Examples of cytokine medium additives are interferons, interleukins or tumor necrosis factor-α (TNFα). Salt solutions may also be added to the media, including Alseverr's Solution, Dulbecco's Phosphate Buffered Saline (DPBS), Earle's Balanced Salt Solution, Gey's Balanced Salt Solution (GBSS), Hanks' Balanced Salt Solution (HBSS), Puck's Saline A, and Tyrode's Salt Solution. If necessary, additives and culture components in different culture conditions be can optimized, as these may alter cell response, activity lifetime or other features affecting bioactivity. In addition,

the surface on which the cells are grown can be coated with a variety of substrates that contribute to survival, growth and/or differentiation of the cells. These substrates include but are not limited to, laminin, EHS-matrix, collagens, poly-L-lysine, poly-D-lysine, polyornithine and fibronectin. When three-dimensional cultures are desired, extracellular matrix gels may be used, such as collagen, EHS-matrix, or gelatin (denatured collagen). Cells may be grown on top of such matrices, or may be cast within the gels themselves.

If desired, the media may be further supplemented with reagents that limit acidosis of the cultures, such as buffer addition to the medium (such as N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BIS-Tris), N-(20hydroxyethyl)piperazine-N'3-propanesulfonic acid (EPPS or HEPPS), glyclclycine, N-2-hydroxyehtylpiperazine-N'-2-ethanesulfonic acid (HEPES), 3-(N-morpholino)propane sulfonic acid (MOPS), Piperazine-N,N'-bis(2-ethane-sulfonic acid) (PIPES), sodium bicarbonate, 3-(N-tris(hydroxymethyl)-methyl-amino)-2-hydroxy-propanesulfonic acid) TAPSO, (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), N-tris(hydroxymethyl)methyl-glycine (Tricine), tris(hydroxymethyl)-aminomethane (Tris), *etc.*). Frequent medium changes and changes in the supplied CO_2 (often approximately 5%) concentration may also be used to control acidosis.

Gases for culture typically are about 5% carbon dioxide and the remainder nitrogen, but optionally may contain varying mounts of nitric oxide (starting as low as 3 ppm), carbon monoxide and other gases, both inert and biologically active. Carbon dioxide concentrations typically range around 5%, but may vary between 2-10%. Both nitric oxide and carbon monoxide, when necessary, are typically administered in very small amounts (*i.e.* in the ppm range), determined empirically or from the literature. The temperature at which the cells will grow optimally can be empirically determined, although the culture temperature will usually be within the normal physiological range of the animal from which the cells were isolated.

Detecting nucleolin: antibody-based methods

Nucleolin can be detected at the protein level in cells, tissue sections, cultured cells and extracts thereof. Immunochemical methods to detect protein expression are

well known and include, but are not limited to, Western blotting, immunoaffinity purification, immunoprecipitation, enzyme-linked immunosorbent assay (ELISA), dot or slot blotting, radioimmunoassay (RIA), immunohistochemical detection, immunocytochemical staining, and flow cytometry. Common procedures and instructions using antibodies have been well addressed (*e.g.*, Harlow and Lane, 1988; Harlow and Lane, 1999). Selected antibodies that are useful for detecting plasma membrane nucleolin are shown in Table 1.

Table 1 Anti-nucleolin antibodies

Antibody	**Source**	**Antigen source**	**Notes**
p7-1A4 mouse *m*onoclonal *anti*body (mAb)	Developmental Studies Hybridoma Bank (University of Iowa; Ames, IA)	*Xenopus laevis* oocytes	IgG_1
sc-8031 mouse mAb	Santa Cruz Biotech (Santa Cruz, CA)	human	IgG_1
sc-9893 goat *p*olyclonal Ab (pAb)	Santa Cruz Biotech	human	IgG
sc-9892 goat pAb	Santa Cruz Biotech	human	IgG
clone 4E2 mouse mAb	MBL International (Watertown, MA)	human	IgG_1
clone 3G4B2 mouse mAb	Upstate Biotechnology (Lake Placid, NY)	dog (MDCK cells)	IgG_{1k}

If additional anti-plasma membrane nucleolin antibodies are desired, they can be produced using well-known methods (Harlow and Lane, 1988; Harlow and Lane, 1999). For example, (pAbs) can be raised in a mammalian host by one or more injections of an immunogen, such as an extracellular domain of surface-expressed nucleolin, and, if desired, an adjuvant. Typically, the immunogen (and adjuvant) is injected in a mammal by a subcutaneous or intraperitoneal injection. The immunogen

may include components such as polypeptides (isolated, non-isolated, or recombinantly produced), cells or cell fractions. Examples of adjuvants include Freund's complete and monophosphoryl Lipid A synthetic-trehalose dicorynomycolate (MPL-TDM). To improve the immune response, an immunogen may be conjugated to a polypeptide that is immunogenic in the host, such as keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin or soybean trypsin inhibitor. Alternatively, pAbs may be made in chickens, producing IgY molecules (Schade *et. al.*, 1996).

mAbs may also be made by immunizing a host or lymphocytes from a host, harvesting the mAb-secreting (or potentially secreting) lymphocytes, fusing those lymphocytes to immortalized cells (*e.g.*, myeloma cells), and selecting those cells that secrete the desired mAb (Goding, 1996). If desired, the mAbs may be purified from the culture medium or ascites fluid by conventional procedures such as protein A-sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, ammonium sulfate precipitation or affinity chromatography (Harlow and Lane, 1988; Harlow and Lane, 1999).

The antibodies may be whole antibodies and fragments or derivatives thereof. For example, when assaying live cells, using F_{ab} fragments will eliminate cross-linking, thus preventing the cells from endocytosing the bound antibodies.

An approach using antibodies to detect the presence of an antigen will include one or more, if not all, of the following steps:

(1) Preparing the entity being tested for plasma membrane nucleolin by washing with buffer or water

(2) Blocking non-specific antibody binding sites

(3) Applying the antibody (*e.g.*, nucleolin)

(4) Detecting bound antibody, either *via* a detectable labeled-secondary antibody that recognizes the primary antibody or a detectable label that has been directly attached to, or associated with, the bound (anti-nucleolin) antibody.

Substrates may be washed with any solution that does not interfere with the epitope structure. Common buffers include saline and biological buffers, such as bicine, tricine, and Tris.

Non-specific binding sites are blocked by applying a protein solution, such as bovine serum albumin (BSA; denatured or native), milk proteins, or in the cases wherein the detecting reagent is a secondary antibody, normal serum or immunoglobulins from a non-immunized host animal whose species is the same origin as the detecting antibody. For example, a procedure using a secondary antibody made in goats would employ normal goat serum (NGS).

The substrate is then reacted with the antibody of interest. The antibody may be applied in any form, such as F_{ab} fragments and derivatives thereof, purified antibody (by affinity, precipitation, *etc.*), supernatant from hybridoma cultures, ascites, serum or recombinant antibodies expressed in recombinant cells. The antibody may be diluted in buffer or media, often with a protein carrier such as the solution used to block non-specific binding sites; the useful antibody concentration is usually determined empirically. In general, polyclonal sera, purified antibodies and ascites may be diluted 1:50 to 1:200,000, more often, 1:200 to 1:500. Hybridoma supernatants may be diluted 1:0 to 1:10, or may be concentrated by dialysis or ammonium sulfate precipitation (or any other method that retains the antibodies of interest but at least partially removes the liquid component and preferably other small molecules, such as salts) and diluted if necessary. Incubation with antibodies may be carried out for as little as 20 minutes at 37° C, 2 to 6 hours at room temperature (approximately 22° C), or 8 hours or more at 4° C.

To detect an antibody-antigen complex, a label may be used. The label may be coupled to the binding antibody, or to a second antibody that recognizes the first antibody, and is incubated with the sample after the primary antibody incubation and thorough washing. Suitable labels include fluorescent moieties, such as fluorescein isothiocyanate; fluorescein dichlorotriazine and fluorinated analogs of fluorescein; naphthofluorescein carboxylic acid and its succinimidyl ester; carboxyrhodamine 6G; pyridyloxazole derivatives; Cy2, 3 and 5; phycoerythrin; fluorescent species of succinimidyl esters, carboxylic acids, isothiocyanates, sulfonyl chlorides, and dansyl chlorides, including propionic acid succinimidyl esters, and pentanoic acid succinimidyl esters; succinimidyl esters of carboxytetramethylrhodamine; rhodamine Red-X succinimidyl ester; Texas Red sulfonyl chloride; Texas Red-X succinimidyl ester; Texas Red-X sodium tetrafluorophenol ester; Red-X; Texas Red dyes;

tetramethylrhodamine; lissamine rhodamine B; tetramethylrhodamine; tetramethylrhodamine isothiocyanate; naphthofluoresceins; coumarin derivatives; pyrenes; pyridyloxazole derivatives; dapoxyl dyes; Cascade Blue and Yellow dyes; benzofuran isothiocyanates; sodium tetrafluorophenols; 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene. Suitable labels further include enzymatic moieties, such as alkaline phosphatase or horseradish peroxidase; radioactive moieties, including ^{35}S and ^{135}I-labels; avidin (or streptavidin)-biotin-based detection systems (often coupled with enzymatic or gold signal systems); and gold particles. In the case of enzymatic-based detection systems, the enzyme is reacted with an appropriate substrate, such as 3, 3'-diaminobenzidine (DAB) for horseradish peroxidase; preferably, the reaction products are insoluble. Gold-labeled samples, if not prepared for ultrastructural analyses, may be chemically reacted to enhance the gold signal; this approach is especially desirable for light microscopy. The choice of the label depends on the application, the desired resolution and the desired observation methods. For fluorescent labels, the fluorophore is excited with the appropriate wavelength and the sample observed using a microscope, confocal microscope, or FACS machine. In the case of radioactive labeling, the samples are contacted with autoradiography film, and the film developed; alternatively, autoradiography may also be accomplished using ultrastructural approaches. Alternatively, radioactivity may be quantified using a scintillation counter.

Cytological-based approaches:

Immunofluorescence/immunohistochemical

Protein expression by cells or tissue can be ascertained by immunolocalization of an antigen. Generally, cells or tissue are preserved by fixation, exposed to an antibody that recognizes the epitope of interest, such as a nucleolin, and the bound antibody visualized.

Any cell, cell line, tissue, or even an entire organism is appropriate for fixation. Cells may be cultured *in vitro* as primary cultures, cell lines, or harvested from tissue and separated mechanically or enzymatically. Tissue may be from any organ, plant or animal, and may be harvested after or prior to fixation. Fixation, if desired, may be by any known means; the requirements are that the protein to be

detected be not rendered unrecognizable by the binding agent, most often an antibody. Appropriate fixatives include paraformaldehyde-lysine-periodate, formalin, paraformaldehyde, methanol, acetic acid-methanol, glutaraldehyde, acetone, Karnovsky's fixative, *etc.* The choice of fixative depends on variables such as the protein of interest, the properties of a particular detecting reagent (such as an antibody), the method of detection (fluorescence, enzymatic) and the method of observation (epifluorescence microscopy, confocal microscopy, light microscopy, electron microscopy, *etc.*). The sample is usually first washed, most often with a biological buffer, prior to fixation. Fixatives are prepared in solution or in biological buffers; many fixatives are prepared immediately prior to applying to the sample. Suitable biological buffers include saline (*e.g.*, phosphate buffered saline), N-(carbamoylmethyl)-2-aminoethanesulfonic acid (ACES), N-2-acetamido-2-iminodiacetic acid (ADA), bicine, bis-tris, 3-cyclohexylamino-2-hydroxy-1-propanesulfonic acid (CAPSO), ethanolamines, glycine, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 2-N-morpholinoethanesulfonic acid (MES), 3-N-morpholinopropanesulfonic acid (MOPS), 3-N-morpholino-2-hyrdoxy-propanesulfonic acid (MOPSO), piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), tricine, triethanolamine, *etc.* An appropriate buffer is selected according to the sample being analyzed, appropriate pH, and the requirements of the detection method. A useful buffer is *p*hosphate *b*uffered *s*aline (PBS). After fixation, the sample may be stored in fixative, preferably fresh, or temporarily or indefinitely, at a temperature between about 4° C to about 22° C.

After fixation from 5 minutes to 1 week, depending on the sample size, sample thickness, and viscosity of the fixative, the sample is washed in buffer. If the sample is thick or sections are desired, the sample may be embedded in a suitable matrix. For cryosectioning, sucrose is infused, and embedded in a matrix, such as OCT Tissue Tek (Andwin Scientific; Canoga Park, CA) or gelatin. Samples may also be embedded in paraffin wax, or resins suitable for electron microscopy, such as epoxy-based (Araldite, Polybed 812, Durcupan ACM, Quetol, Spurr's, or mixtures thereof; Polysciences, Warrington, PA), acrylates (London Resins (LR White, LR gold), Lowicryls, Unicryl; Polysciences), methylacrylates (JB-4, OsteoBed; Polysciences), melamine (Nanoplast; Polysciences) and other media, such as DGD, Immuno-Bed

(Polysciences) and then polymerized. Resins that are especially appropriate include hydrophilic (such as Lowicryls, London Resins, water-soluble Durcupan, *etc.*) since these are less likely to denature the protein of interest during polymerization and will not repel antibody solutions. When embedded in wax or resin, samples are dehydrated by passing them through a concentration series of ethanol or methanol; in some cases, other solvents may be used, such as polypropylene oxide. Embedding may occur after the sample has been reacted with the detecting agents, or samples may be first embedded, sectioned (via microtome, cyrotome, or ultramicrotome), and then the sections reacted with the detecting reagents. In some cases, the embedding material may be partially or completely removed before detection to facilitate antigen access.

In some instances, the nucleolin epitope(s) to which the antibody binds may be rendered unavailable because of fixation. Antigen retrieval methods can be used to make the antigen available for antibody binding. Many recourses are available (reviewed in, for example, McNicol and Richmond, 1998; Robinson and Vandre, 2001; Shi *et al.*, 2001). Common methods include using heat supplied from autoclaves, microwaves, hot water or buffers, pressure cookers, or other sources of heat. Often the sources of heat are used in sequence; the samples must often be in solution (*e.g.*, microwave treatments). Detergent treatment may also unmask antigens, such as sodium dodecyl sulfate (SDS, 0.25% to 1%) or other denaturing detergents. Chemical methods include strong alkalis (such as NaOH), prolonged immersion in water, urea, formic acid and refixation in zinc sulfate-formalin. In other instances, proteolytic enzyme treatment will modify the antigen such that it is available to the antibody. Any number of proteases may be used, such as trypsin. These methods may be combined to achieve optimal results. The choice of the antigen retrieval method will depend on the sample, its embedment (if any), and the anti-nucleolin antibody.

Especially in the cases of immunofluorescent or enzymatic product-based detection, background signal due to residual fixative, protein cross-linking, protein precipitation or endogenous enzymes may be quenched, using, *e.g.*, ammonium chloride or sodium borohydride or a substance to deactivate or deplete confounding endogenous enzymes, such as hydrogen peroxide which acts on peroxidases. To

detect intracellular proteins in samples that are not to be sectioned, samples may be permeabilized. Permeabilizing agents include detergents, such as t-octylphenoxypolyethoxyethanols, polyoxyethylenesorbitans, and other agents, such as lysins, proteases, *etc.*

Non-specific binding sites are blocked by applying a protein solution, such as bovine serum albumin (BSA; denatured or native), milk proteins, or preferably in the cases wherein the detecting reagent is an antibody, normal serum or IgG from a non-immunized host animal whose species is the same is the same origin of the detecting antibody.

Flow cytometry/*F*luorescence-*A*ctivated *C*ell *S*orting (FACS)

Methods of performing flow cytometry are well known (Orfao and Ruiz-Arguelles, 1996). Because plasma membrane nucleolin is being probed, cell permeabilization that allows access to cytoplasmic compartments is undesirable. After harvesting, cells are prepared as a single-cell suspension; cells are then incubated with an anti-nucleolin antibody usually after blocking non-specific binding sites. Preferably, the anti-nucleolin antibody is labeled with a fluorescent marker. If the antibody is not labeled with a fluorescent marker, a second antibody that is immunoreactive with the first antibody and contains a fluorescent marker is used. After sufficient washing to ensure that excess or unbound antibodies are removed, the cells are ready for flow cytometry.

Biochemical-based approaches:

In these approaches, it is first desirable to isolate plasma membrane proteins from other cellular compartments. This may be done in any number of ways, such as simple cell extraction, differential extraction or mechanical disruption followed by separation of cellular compartments on gradients (such as sucrose or polydextran) by centrifugation, extraction followed by immunoselecting appropriate cellular compartments with plasma membrane-specific antibodies, *etc.* An example of such an approach is described in Naito *et al.* (1988) and Yao *et al.* (1996b). Extracting reagents are well known. For examples, solvents such as methanol may be occasionally useful. More likely, detergents, such as t-octylphenoxypolyethoxyethanol (also known as polyethylene glycol tert-octylphenyl

ether) are particularly useful for simple extractions. Also useful are glucopyranosides, maltopyranosides, maltosides, polyoxyethylene esters, other polyoxyethylene ethers, salts of alginic, caprylic, cholic 1-decanesulfonic, deoxycholic, dioctyl sulfosuccinate, 1-dodecanesulfonic, glyocholic, glycodeoxycholic, 1-heptanesulfonic, 1-hexanesulfonic, N-lauroylsacrosine, lauryl sulfate (*e.g.*, SDS), 1-nonanesulfonic, 1-octanesulfonic, 1-pentanesulfonic, taurocholic and tauodexycholic acids; sodium 7-ethyl-2-methyl-4-undecyl sulfate, and sodium 2-ethylhexyl sulfate. Other useful detergents include(3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulfonate, N-decyl-, N-dodecyl-, N-hexadecyl-, N-octadecyl-, N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonates and phosphatidylcholine. Less useful, but may be helpful in some cases, are alkyltrimethylammonium bromides, benzalkonium chloride, benzethonium chloride, benzyldimethyldodecylammonium bromide, benzyldimethylhexadecylammonium chloride, cetyldimethylethylammonium bromide, cetylpyridinium, decamethonium bormide, dimethyldioctadecylammonium bromide, methylbenzethonium chloride, methyltiroctylammonium chloride, and N,N',N'-polyoxyehtlylene(10)-N-tallow-1,3-diaminopropane. The different extracting reagents may be used singly or in combination; they may be prepared in simple aqueous solutions or suitable buffers.

Polyethylene glycol ter-octylphenyl ether is particularly useful for differential extraction by taking advantage of the low cloud point to separate membrane proteins from soluble proteins into two different phases.

Extraction buffers may contain protease inhibitors, such as aprotinin, benzamidine, antipain, pepstatin and iodoacetamide.

Extracts are then assayed for nucleolin expression. For those techniques that separate surface plasma membrane from other cellular components (especially the nucleus), the nucleolin detecting agents need not be specific for extracellular plasma membrane nucleolin epitopes.

Immunosorbent assay (ELISA) (Ausubel *et al.*, 1987)

Various types of enzyme linked immunosorbent assays (ELISAs) to detect protein expression are known, and these are applicable to nucleolin detection.

However, other ELISA-like assays include radio-immunoassays and other non-enzyme linked antibody binding assays and procedures. In these assays, the cell surface proteins are the principle components in the cell preparation.

The double antibody-sandwich ELISA technique is especially useful. The basic protocol for a double antibody-sandwich ELISA is as follows: A plate is coated with anti-nucleolin antibodies (capture antibodies). The plate is then washed with a blocking agent, such as BSA, to block non-specific binding of proteins (antibodies or antigens) to the test plate. The test sample is then incubated on the plate coated with the capture antibodies. The plate is then washed, incubated with anti-nucleolin antibodies, washed again, and incubated with a specific antibody-labeled conjugates and the signal appropriately detected.

In other ELISAs, proteins or peptides are immobilized onto a selected surface, the surface exhibit may have affinity for proteins, such as the wells of a specially-treated polystyrene microtiter plate. After washing to remove incompletely adsorbed material, one would then generally desire to bind or coat with a nonspecific protein that is known to be antigenically neutral with anti-nucleolin antibodies, such as BSA or casein, onto the well bottom. This step allows for blocking of nonspecific adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antibodies onto the surface. When the antibodies were created in an animal by conjugating a polypeptide to a protein (*e.g.*, BSA), a different protein is usually used as a blocking agent, because of the possibility of the presence of antibodies to the blocking protein the antibody composition.

After binding of nucleolin to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with an anti-nucleolin antibody composition in a manner conducive to immune complex (antigen/antibody) formation. Such conditions include diluting the antibody composition with diluents such as BSA, bovine γ globulin (BGG) and PBS/Polyoxyethylenesorbitan monolaurate. These added agents also assist in the reduction of nonspecific background signal. The layered antibody composition is then allowed to incubate for, *e.g.*, from 2 to 4 hours at 25° C to 37° C. Following incubation, the antibody composition-contacted surface is washed so as to

remove non-immunocomplexed material. One washing procedure includes washing with a PBS/polyoxyethylenesorbitan monolaurate or borate buffer solution.

Following formation of specific immunocomplexes between the test sample and the antibody and subsequent washing, immunocomplex formation is detected using a second antibody having specificity for the anti-nucleolin antibody. For detection, the secondary antibody is associated with detectable label, such as an enzyme or a fluorescent molecule. A number of immunoassays are discussed in U.S. Patent Nos. 5,736,348, 5,192,660, and 4,474,892.

Western blotting (Ausubel *et al.*, 1987)

Western blotting methods are well known. Generally, a protein sample is subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at such conditions as to yield an appropriate separation of proteins within the sample. The proteins are then transferred to a membrane (*e.g.*, nitrocellulose, nylon, *etc.*) in such a way as to maintain the relative positions of the proteins to each other.

Visibly labeled proteins of known molecular weight may be included within a lane of the gel. These proteins serve as a control to insure adequate transfer of the proteins to the membrane, as well as molecular weight markers for determining the relative molecular weight of other proteins on the blot. Alternatively, unlabeled marker proteins (or in some rare instances, no marker proteins) are detected after transfer with Brilliant Blue (G or R; Sigma; St. Louis, MO) other protein dyes. After protein transfer, the membrane is submersed in a blocking solution to prevent nonspecific binding of the primary antibody.

The primary antibody, *e.g.*, anti-nucleolin, may be labeled and the presence and molecular weight of the antigen may be determined by detecting the label at a specific location on the membrane. However, the primary antibody may not be labeled, and the blot is further reacted with a labeled second antibody. This secondary antibody is immunoreactive with the primary antibody; for example, the secondary antibody may be one to rabbit imunoglobulins and labeled with alkaline phosphatase. An apparatus for and methods of performing Western blots are described in U.S. Patent No. 5,567,595.

Immunoprecipitation (Ausubel *et al.*, 1987; Harlow and Lane, 1999)

Protein expression can be determined and quantified by isolating antigens using immunoprecipitation. Methods of immunoprecipitations are described in U.S. Patent No. 5,629,197. Immunoprecipitation involves the separation of the target antigen component from a complex mixture and is used to discriminate or isolate minute amounts of protein. For the isolation of cell-surface proteins, nonionic salts are often used.

For example, an immunoprecipitation from whole cells may be performed as follows. Cells are extracted with one or more detergents (see above), such as, for example, 1% t-octylphenoxypolyethoxyethanol/0.1% SDS/150 mM NaCl in 20 mM Tris buffer, pH 8.6. After extraction, which may be aided by agitation, insoluble debris is removed using a centrifuge. Anti-nucleolin antibody is added to the extracts, and then the samples are incubated 30 minutes to overnight at 4° C. *Staphylococcus aureus* or recombinantly-produced Protein A or Group C *Staphylococcus* Protein G conjugated to sepharose or tris-acryl beads are then added. In those instances when the anti-nucleolin antibody does not bind well to Protein A, IgG Abs that recognize antibodies of the animal in which the anti-nucleolin antibody was made is simultaneously added. The samples are then incubated with gentle agitation for around 2 hours at 4° C. The beads or bacterial cells, now bound to the antibody-antigen complexes, are thoroughly washed, usually first with either the extraction solution or a high salt buffer, then a salt-less buffer or water to remove nonspecifically-bound proteins and residual detergent molecules. After removing residual buffer, the beads are incubated with a buffer, such as electrophoresis sample buffer, and then subjected to 95°C for 3-5 minutes to elute bound proteins from the beads. The samples are then ready for analysis and nucleolin detection.

Other methods:

Immunoselection procedures (other than FACS) (Ausubel *et al.*, 1987)

Cells expressing plasma membrane nucleolin can be easily isolated by "panning" on plastic plates coated with anti-antibody antibodies (Wysocki and Sato, 1978). Panning has many advantages over other immunoselection procedures: It is fast, efficient (10^7 cells can easily be panned on two 60-mm plastic plates in 30 minutes), and inexpensive.

In general, a single cell suspension is labeled with an anti-nucleolin antibody, and then is incubated on a substrate coated with a secondary antibody (with non-specific binding sites blocked). After 1 to 3 hours incubation at room temperature, non-adherent cells are washed away. In this embodiment, bound cells indicate that nucleolin is expressed in the plasma membrane, indicating a neoplastic cell.

Detecting nucleolin: Oligonucleotide-based methods

GROs and other oligonucleotides that recognize and bind nucleolin (Bates *et al.*, 1999; Miller *et al.*, WO 00/61597, 2000; Xu *et al.*, 2001) can be used much the same way as antibodies are. Examples of suitable assays are given below. In some cases, incorporating the GRO nucleotides into larger nucleic acid sequences may be advantageous; for example, to facilitate binding of a GRO nucleic acid to a substrate without denaturing the nucleolin-binding site.

Useful GROs that bind nucleolin (and also have the biological property of inhibiting cancer cell growth) have been described (Bates *et al.*, 1999; Miller *et al.*, WO 00/61597, 2000; Xu *et al.*, 2001). They include those shown in Table 2. Control GROs are useful for detecting background signal levels.

Table 2 Non-antisense GRO that bind nucleolin and non-binding controls[1,2,3]

GRO	Sequence	SEQ ID NO:
GRO29A[1]	tttggtggtg gtggttgtgg tggtggtgg	1
GRO29-2	tttggtggtg gtggttttgg tggtggtgg	2
GRO29-3	tttggtggtg gtggtggtgg tggtggtgg	3
GRO29-5	tttggtggtg gtggtttggg tggtggtgg	4
GRO29-13	tggtggtggt ggt	5
GRO14C	ggtggttgtg gtgg	6
GRO15A	gttgtttggg gtggt	7
GRO15B[2]	ttgggggggg tgggt	8
GRO25A	ggttggggtg ggtggggtgg gtggg	9
GRO26B[1]	ggtggtggtg gttgtggtgg tggtgg	10
GRO28A	tttggtggtg gtggttgtgg tggtggtg	11
GRO28B	tttggtggtg gtggtgtggt ggtggtgg	12
GRO29-6	ggtggtggtg gttgtggtgg tggtggttt	13
GRO32A	ggtggttgtg gtggttgtgg tggttgtggt gg	14
GRO32B	tttggtggtg gtggttgtgg tggtggtggt tt	15
GRO56A	ggtggtggtg gttgtggtgg tggtggttgt ggtggtggtg gttgtggtgg tggtgg	16

GRO	Sequence	SEQ ID NO:
CRO	tttcctcctc ctccttctcc tcctcctcc	18
GRO A	ttagggttag ggttagggtt aggg	19
GRO B	ggtggtggtg g	20
GRO C	ggtggttgtg gtgg	21
GRO D	ggttggtgtg gttgg	22
GRO E	gggttttggg	23
GRO F	ggttttggtt ttggttttgg	24
GRO G[1]	ggttggtgtg gttgg	25
GRO H[1]	ggggttttgg gg	26
GRO I[1]	gggttttggg	27
GRO J[1]	ggggttttgg ggttttgggg ttttgggg	28
GRO K[1]	ttggggttgg ggttggggtt gggg	29
GRO L[1]	gggtgggtgg gtgggt	30
GRO M[1]	ggttttggtt ttggttttgg ttttgg	31
GRO N[2]	tttcctcctc ctccttctcc tcctcctcc	32
GRO O[2]	cctcctcctc cttctcctcc tcctcc	33
GRO P[2]	tggggt	34
GRO Q[2]	gcatgct	35

GRO	Sequence	SEQ ID NO:
GRO R[2]	gcggtttgcg g	36
GRO S[2]	tagg	37
GRO T[2]	ggggttgggg tgtggggttg ggg	38

[1]Indicates a good plasma membrane nucleolin-binding GRO. [2]Indicates a nucleolin control (non-plasma membrane nucleolin binding).

[3]GRO sequence without [1] or [2] designations have some anti-proliferative activity.

Cytological-based approaches:

Cellular localization/labeling (relative of immuno-based localization/labeling assays)

The procedures outlined above for the immuno-based localization assays (such as immunofluorescence or FACS) are also applicable to those assays wherein the detecting reagent is a nucleolin-binding GRO. Modifications include those to prevent non-specific binding, using denatured DNA, such as from salmon sperm instead of a protein such as BSA. For detection, similar labels as outlined above are also useful as long as the GRO can be derivatized with the label in some form. For this purpose, biotin-avidin nucleic acid labeling systems are especially convenient, as are digoxigenin ones (Ausubel *et al.*, 1987). The synthesis of biotinylated nucleotides has been described (Langer *et al.*, 1981). Biotin, a water-soluble vitamin, can covalently attached to the C5 position of the pyrimidine ring via an alylamine linker arm; biotin non-covalently binds avidin or streptavidin, which may be easily labeled. Alternatively, biotin is added to oligonucleotides during synthesis by coupling to the 5'-hydroxyl of the terminal nucleotide. Digoxigenin-11-dUTP can be incorporated into DNA by either nick translation or random oligonucleotide-primed synthesis protocols. Digoxigenin is detected using labeled anti-digoxigenin antibodies. Convenient digoxigenin systems are commercially available (Roche Molecular Biochemicals; Indianapolis, IN). An example of a procedure using oligonucleotides to detect and localize proteins has been described by Davis *et al.*, 1998.

Biochemical-based approaches:

GROs may also be used in a similar fashion as antibodies to detect nucleolin in biochemical approaches, as described above. For example, "Southwestern"-type blotting experiments may be performed with GROs (Bates *et al.*, 1999; Miller *et al.*, WO 00/61597, 2000). After cells have been appropriately extracted (for example, differentially to separate plasma membrane proteins from intracellular proteins), the proteins are subjected to electrophoresis on polyacrylamide gels and transferred to a substrate, such as a polyvinliden difluoride membrane. Proteins are denatured and renatured by washing for 30 minutes at 4° C with 6 M gaunidine-HCl, followed by washes in 3 M, 1.5 M and 0.75 M guanidine HCl in 25 mM HEPES (pH 7.9)/4 mM KCl/3 mM $MgCl_2$). After blocking non-specific binding sites with 5% non-fat dried

milk in HEPES buffer, the labeled GRO is hybridized for 2 hours at 4° C in HEPES binding buffer supplemented with 0.25% NDM, 0.05% NP-40, 400 ng/ml salmon sperm DNA and 100 ng/ml of an unrelated mixed sequence oligonucleotide, such as tcgagaaaaa ctctcctctc cttccttcct ctcca; SEQ ID NO:17. After washing with HEPES binding buffer, the signal is detected appropriately.

Other methods:

Arrays

Arrays of immobilized nucleolin-binding reagents on chips

A chip is an array of regions containing immobilized molecules, separated by regions containing no molecules or immobilized molecules at a much lower density. For example, a protein chip may be prepared by applying nucleolin-binding antibodies; an "aptamer"-like chip may be prepared by applying nucleolin binding GROs. The remaining regions are left uncovered or are covered with inert molecules. The arrays can be rinsed to remove all but the specifically immobilized polypeptides or nucleic acids. In addition, chips may also be prepared containing multiple nucleolin-binding antibodies (Table 1), nucleic acids (such as GROs; Table 2), or both, and may contain control antibodies and/or nucleic acids that are non-reactive with nucleolin. Such an array would allow for simultaneous test confirmation, duplication and internal controls.

Proteins, such as anti-nucleolin antibodies, can be immobilized onto solid supports by simple chemical reactions, including the condensation of amines with carboxylic acids and the formation of disulfides. This covalent immobilization of proteins on inert substrates can prevent high background signals due to non-specific adsorption. Substrates derivatized with other molecules, such as biotin, are also useful when the protein to be immobilized is derivatized with avidin or streptavidin, or *vice-versa*. In some rare cases, especially when anti-nucleolin antibody-encoding nucleic acid sequences are available, fusion polypeptides comprising anti-nucleolin antibody may be advantageous for immobilization onto a substrate.

The surface may be any material to which a the nucleolin binding agent can be immobilized. For example, the surface may be metal, glass, ceramic, polymer, wood or biological tissue. The surface may include a substrate of a given material and a

layer or layers of another material on a portion or the entire surface of the substrate. The surfaces may be any of the common surfaces used for affinity chromatography, such as those used for immobilization of glutathione for the purification of GST fusion polypeptides. The surfaces for affinity chromatography include, for example, sepharose, agarose, polyacrylamide, polystyrene and dextran. The surface need not be a solid, but may be a colloid, an exfoliated mineral clay, a lipid monolayer, a lipid bilayer, a gel, or a porous material.

The immobilization method desirably controls the position of the nucleolin binding agent on the surface; for example, enabling the antigen binding portions of antibodies unattached to the substrate, while the non-antigen binding portions are rooted to the substrate. By controlling the position of individual reactant ligands, patterns or arrays of the ligands may be produced. The portions of the surface that are not occupied by the nucleolin-binding reagent do not allow non-specific adsorption of polypeptides or polynucleotides.

In this embodiment, a sample from a subject, for example, blood, is passed over a chip containing nucleolin-binding molecules. A biosensing device, such as machine that detects changes in surface plasmon resonance, is then used to detect bound nucleolin. BIAcore (Uppsala, Sweden) chips serve as examples of useful chips and detection machines.

Prognostic assays

Diagnostic methods can furthermore be used to identify subjects having, or at risk of developing, a neoplasia at an early stage of disease development, since the surface expression of nucleolin can be detected earlier than in conventional methods. Prognostic assays can be used to identify a subject having or at risk for developing a neoplasia, such as a subject who has a family history of harmful neoplasias, especially cancers. A method for identifying such an individual would include a test sample obtained from a subject and testing for cell surface localization of nucleolin.

In another embodiment, detecting plasma membrane nucleolin and then either qualitatively or quantitatively assessing the amount of nucleolin (usually indirectly through the signal generated from bound nucleolin molecules) can indicate the rate of

cell proliferation, since plasma membrane nucleolin levels correlate with cell proliferation rates.

Kits

Kits, containers, packs, or dispensers containing nucleolin probes and detection reagents, together with instructions for administration, may be assembled. When supplied as a kit, the different components may be packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage without losing the active components' functions.

Kits may also include reagents in separate containers that facilitate the execution of a specific test, such as diagnostic tests. For example, non-nucleolin-binding GROs may be supplied for internal negative controls, or nucleolin and a nucleolin-binding reagent for internal positive controls. The components of a kit are an anti-nucleolin agent used to probe for nucleolin, a control sample, and optionally a composition to detect nucleolin. Examples of anti-nucleolin agents include an anti-nucleolin antibody (*e.g.*, as shown in Table 1) or fragment thereof; if labeled, then a nucleolin-binding detection reagent is superfluous. A nucleolin-binding oligonucleotide (*e.g.*, as shown in Table 2), which may be derivatized such that a second labeled reagent may bind (such as biotin). However, if a labeled GRO nucleic acid is provided, then a second labeled reagent is superfluous. Examples of detection reagents include: labeled secondary antibodies, for example, an anti-mouse pAb made in donkey and then tagged with a fluorophore such as rhodamine, or a labeled reagent to detect oligonucleotides such as GROs; for example, avidin or streptavidin linked to horseradish peroxidase when the probe is biotinylated. Control components may include: normal serum from the animal in which a secondary antibody was made; a solution containing nucleolin polypeptide or nucleolin binding oligonucleotide; a dot blot of nucleolin protein to assay nucleolin-binding reagent reactivity; or fixed or preserved cells that express nucleolin in the plasma membrane. Other components may include buffers, fixatives, blocking solutions, microscope slides and/or cover slips or other suitable substrates for analysis, such as microtiter plates; detergent or detergent solutions or other cell extraction reagents; miscellaneous

reagents, protease inhibitors, various containers and miscellaneous tools and equipment to facilitate the assays.

(a) *Containers or vessels*

The reagents included in the kits can be supplied in containers of any sort such that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain lyophilized nucleolin binding reagents (such as anti-nucleolin antibodies or nucleolin-binding oligonucleotides) or buffers that have been packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers (*i.e.*, polycarbonate, polystyrene, *etc.*), ceramic, metal or any other material typically employed to hold reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes that may have foil-lined interiors, such as aluminum or alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to mix. Removable membranes may be glass, plastic, rubber, *etc.*

(b) *Instructional materials*

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, DVD, videotape, audio tape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

The following examples are intended to illustrate the present invention without limitation.

EXAMPLES

Example 1 Immunofluorescent labeling of plasma membrane nucleolin in cells

This example illustrates a procedure that stains nuclear nucleolin, or only plasma membrane nucleolin.

Cells from the cell lines DU145 (human prostrate cancer), MDA-MB-231 (human breast cancer) HeLa (human cervical cancer) and HS27 (normal skin fibroblasts) (all available from ATCC; Manassas, VA) were released from culture substrates with trypsin, resuspended into single cells and plated onto microscope slides. The slides seeded with cells were incubated at 37° C until they were well attached, as assayed by visual inspection using a microscope. After rinsing the attached cells once with PBS for two minutes, they were fixed in 4% formaldehyde/PBS for at least 15 minutes at 22° C. For nuclear nucleolin staining, cells are permeabilized with 1% Triton X-100 prior to contacting with antibody. After washing twice with PBS, 5 minutes/wash, non-specific binding sites were blocked for 15-60 minutes with 1% NGS/PBS at 22° C, and then incubated with mouse anti-nucleolin antibodies diluted in 1% NGS/PBS or PBS/Tween (0.05%-0.1%) for 1 hour to overnight at 4° C. The samples were washed four times, 5 minutes each with PBS, and then incubated with goat anti-mouse pAb labeled with FITC-labeled secondary antibodies diluted in PBS for 1 hour at 22°C. After again washing four times with PBS for 5 minutes each, the samples were mounted in Mowiol mounting media (prepared as follows: 9 ml/glycerol and 3.36 g Mowiol 40-88 were agitated forl h at 22° C. Then, 9 ml of water was then added, and agitation continued for 2 h at 22° C. Tris (0.2 M, pH 8.5; 18 ml) was then added, and the solution incubated for 6 h at 50° C until the solids were almost completely dissolved. After centrifugation at 5,000 x *g*, the liquid phase was used for mounting), observed under a microscope, and photographed.

Figures 1 and 2 show nuclear (Figure 1) and plasma membrane (Figure 2) nucleolin staining in the various cell lines. Shown are immunofluorescent (Figures 1 and 2; panels B, D, F, H) and parallel phase contrast micrographs (Figures 1 and 2; panels A, C, E, G); DU145 cells are shown in A and B; MDA-MB-231 cells are shown in C and D; HeLa cells are shown in E and F; and HS27 cells are shown in G and H. All cell lines show clear nuclear nucleolin staining (Figures 1A, 1C, 1E and 1G). Note that when the cells are not permeabilized, thus restricting antibody access to the surface plasma membrane, the normal skin cell line, HS27, is completely

negative for plasma membrane staining (Figure 2H) while cancer cells show strong staining (Figures 2B, 2D, 2F and 2H). Staining plasma membrane nucleolin is thus a superior method for diagnosis and prognosis compared to nuclear nucleolin or silver-staining NORs.

Example 2 Correlation of the degree of plasma membrane nucleolin expression and cancer aggressiveness

This experiment demonstrates that cell lines with high levels of plasma membrane nucleolin correspond to those with the most rapid proliferation.

Two cancer cell lines, DU145 and HeLa, and one normal cell line, HS27, were assayed for proliferation rate and compared. Cell doubling time is calculated by determining cell density at regular intervals using the MTT assay (based upon the ability of living cells to reduce 3-(van de Loosdrecht *et al.*, 1994)-2,5 diphenyltetrazolium bromide (MTT) into formazan; (van de Loosdrecht *et al.*, 1994)), and confirmed by counting the cells using trypan blue exclusion.

Figure 3 shows the comparative proliferation rates of DU145 (squares), HeLa (diamonds) and HS27 (circles) as measured by MTT assay. Until 3 days of culture, growth rates are similar, but after 3 days, HeLa and DU145 increase at a faster rate than the normal HS27 cells. Although MDA-MB-231 was not included in this experiment, proliferation rate has been determined to be DU145 > MDA-MB-231 > HeLa > HS27. Note that the cell lines with high levels of plasma membrane nucleolin (see Figure 2) correspond to those with the most rapid proliferation (DU145 and HeLa).

Example 3 Immunofluorescent labeling of nucleolin in paraffin-embedded tissue sections

This example provides a suitable technique to detect and localize nucleolin in a fixed sample that has been embedded.

Sections of cells fixed and embedded in paraffin wax and anchored on microscope slides were washed in three changes of xylene (2 minutes each) to remove the paraffin, hydrated in graded alcohols (series 100%, 95% and 70%; 2 minutes each), and placed in PBS for 5 minutes. Antigen recovery used the approach of *l*ow

*t*emperature *a*ntigen *r*etrieval (LTAR; (Shi *et al.*, 1997; Shi *et al.*, 2001)): After digestion with 0.1% trypsin-EDTA (v/v) (Invitrogen Corp.; Carlsbad, CA) diluted in PBS for 15 minutes at 37° C/5% CO_2, the samples were washed with deionized water and incubated in 10 mM citrate buffer (pH 6) for 2 hours at 80° C. After cooling, the slides were rinsed with deionized water and then PBS.

Non-specific binding sites were blocked by incubation in 3% BSA in PBS for 30 minutes at 22° C. The samples were then incubated with 4 μg/ml mouse anti-nucleolin mAb (Santa Cruz) diluted in PBS/1% NGS at 4° C overnight. The samples were then brought to 22° C, washed four times with PBS for 5 minutes, and then reacted with 50 μg/ml Alexa488-conjugated goat anti-mouse IgG (Molecular Probes; Eugene, OR) and 2 μg/ml propidium iodide diluted in PBS/1% NGS for 1 hour at 22° C. After washing four times with PBS for 5 minutes, the samples were mounted in Mowiol mounting medium and observed under a fluorescent microscope.

Figure 4 shows the results of such an experiment. A clinical sample of a squamous cell carcinoma of the head and neck was prepared and probed for plasma membrane nucleolin. Plasma membrane nucleolin signal was relegated to malignant, neoplastic cells. Figure 4A shows the immunofluorescent signal obtained from probing for nucleolin; the nuclei are counterstained with a DNA-intercalating dye. Figure 4B shows a parallel phase contrast micrograph. Figures 4C and 4D are duplicates of Figures 4A and 4B, except markings have been added to better indicate areas of staining. In region 1, the signal is strong on the cells (faint signal in relation to the nuclear staining in Fig. 4A); these cells are in loosely-organized tissue and are less densely-packed, suggesting that they are malignant. In region **2**, normal cells (as delineated by well-packed cells and organized tissue), cells display no plasma membrane nucleolin signal.

Example 4 Plasma membrane nucleolin expression in lung carcinoma cells

This example demonstrates that lung carcinoma cells can be easily identified by staining for plasma membrane nucleolin.

NCI-H1299 (non-small cell lung carcinoma isolated from *H. sapiens* lymph node; (Giaccone *et al.*, 1992; Lin and Chang, 1996)) and NCI-H82 (small cell lung carcinoma cells, *H. sapiens*, (Carney *et al.*, 1985; Little *et al.*, 1983; Takahashi *et al.*,

1989)) cells were released from culture substrates with trypsin, resuspended into single cells and plated onto microscope slides. The cells were incubated at 37° C until they were well-attached as assayed by visual inspection using a microscope. After rinsing the cells once with PBS for 2 minutes, they were fixed in 4% formaldehyde/PBS for at least 15 minutes at 22° C. After washing twice with PBS, 5 minutes/wash, non-specific binding sites were blocked for 15-60 minutes with 1% NGS/PBS at 22° C, and then incubated with mouse anti-nucleolin antibodies for 1 hour to overnight at 4° C. The samples were washed four times, 5 minutes each with PBS and then incubated with goat anti-mouse pAb labeled with FITC-labeled secondary antibodies diluted in PBS with propidium iodide (to stain nuclei) for 1 hour at 22°C. After again washing four times with PBS for 5 minutes each, the samples were mounted in Mowiol mounting media, observed under a microscope and photographed.

Figure 5 shows whole cells probed for plasma membrane nucleolin of the two lung cancer cell lines, NCI-H82 (Figure 5A; a parallel phase contrast image is shown in 5B) and NCI-H1299 (Figure 5C; a parallel phase contrast image is shown in 5D). In both cell lines, plasma membrane nucleolin staining is strong; examples of well-stained cells are denoted by asterisk (*) in Figures 5A and 5C.

Example 5 Plasma membrane nucleolin staining of clinical specimens

To test the feasibility of using this novel method of assaying plasma membrane nucleolin to diagnose tumor, pre-malignant and malignant cells, clinical specimens from healthy subjects and those suffering from a cancer were collected. Samples from peripheral blood, bone marrow and tumor biopsy samples were obtained and stained for plasma membrane nucleolin as described in Example 4. Figure 6 shows phase contrast (B, D, F) and immunofluorescent images (A, C, E) of peripheral blood (A, B) or bone marrow (C, D and E, F). Highly stained cells for plasma membrane nucleolin are marked with an asterisk (*); these were only seen in those patients suffering from carcinomas (A,B and C,D), while cells from a healthy patient did not display any plasma membrane staining (E, F).

Example 6 (Prophetic) Correlation of the degree of plasma membrane nucleolin expression and cancer aggressiveness

Thirty-three lung carcinoma cell lines are analyzed, mostly available from the American Type Culture Collection (Manassas, VA). Cell doubling time is calculated by determining cell density at regular intervals using the MTT assay and confirmed by counting the cells using trypan blue exclusion. In each experiment HeLa cells (Gey *et al.*, 1952) are included as an internal control. Each value is determined from at least two independent experiments with triplicate samples. To determine levels of nuclear and plasma membrane nucleolin, two methods are implemented. First, nuclear and plasma membrane extracts are prepared from each cell line using methods that have as described (Ausubel *et al.*, 1987; Bates *et al.*, 1999; Yao *et al.*, 1996a). Briefly, cells are harvested and resuspended in a hypotonic buffer, then allowed to swell on ice for several minutes. Cells are lysed using a Dounce homogenizer, and nuclei are collected by centrifugation. Nuclei are resuspended in a high salt buffer to extract nuclear proteins; salt is then removed by dialysis. Plasma membrane proteins can be isolated from the S-100 fraction and are separated from cytosolic proteins and other organelles by centrifugation through a sucrose gradient. Nuclear and PM extracts from different cell are analyzed by Western blot analysis (Ausubel *et al.*, 1987) using an anti-nucleolin antidody (Santa Cruz) followed by chemiluminescent visualization. Nucleolin levels are then quantified by densitometry of the resulting signal recorded on X-ray film and normalized to the intensity of HeLa extract controls. The second approach to determine nucleolin levels involves immunofluorescent probing of the cell lines for nucleolin. Cells are probed for nucleolin surface expression in parallel with DU145 cells (Mickey *et al.*, 1977; Stone *et al.*, 1978) as a positive control, HS27 cells as a negative control and HeLa cells as a reference (see Figure 2). Cells are photographed and ranked in order of degree of signal, which may also be quantified (using systems that use software and images to quantitate pixels; in this instance, video images are used)or qualitatively evaluated. The data are then subjected to statistical analysis to demonstrate correlations with the degree of cell proliferation (higher rates of cell proliferation indicate more aggressive cancer cells) with the intensity of nucleolin signal across the entire sample and within subsets.

Example 7 (Prophetic) Lung cancer detection

In this example, patient biopsies, sputum samples and resected lung tissue are probed for plasma membrane nucleolin, and these results are compared to other diagnostic and prognostic markers for lung cancer, utilizing archival and routine clinical specimens for this study.

Methods

Specimens including bronchial biopsies, sputum samples, and resected lung tissue are obtained from human subjects, both healthy and those suffering from lung cancer, and each sample encoded such that at the time of nucleolin probing and observation, the sample origin is unknown.

Probing these samples using immunohistochemical techniques are then implemented. For example, plasma membrane nucleolin is probed with one or more anti-nucleolin Abs selected from Table 1, a signal generated from a flourophore-tagged secondary Ab, and the samples observed and photographed. Appropriate controls include probing with the secondary antibody only, probing with no antibodies, probing with pre-immune serum only, and probing with an antibody known not to react with the cell types being analyzed. To facilitate visualization and localization determination, the cells can be counterstained with Hoechst 33258 or propidium iodide (to visualize nuclei) and/or with fluorescent-tagged phalloidin or phallicidin (to visualize the actin cytoskeleton). The samples are observed, scored (surface signal indicating plasma membrane nucleolin expression) and documented.

REFERENCES

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, *et al.* 1987. Current protocols in molecular biology. John Wiley & Sons, New York.

Babkes, M., A. Ranford, and K. Yaeger. US Patent 6,325,785. 2001. Sputum trap manifold with nested caps.

Bandman, O., H. Yue, N. Corley, and P. Shah. US Patent No. 5,932,475. 1999. Human nucleolin-like protein.

Bates, P.J., J.B. Kahlon, S.D. Thomas, J.O. Trent, *et al.* 1999. Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. *J Biol Chem.* 274:26369-77.

Byrne, C.J. 1986. Laboratory tests : implications for nursing care. Addison-Wesley Pub. Co. Health Sciences Division, Menlo Park, Calif. xxi, 756 pp.

Callebaut, C., J. Blanco, N. Benkirane, B. Krust, *et al.* 1998. Identification of V3 loop-binding proteins as potential receptors implicated in the binding of HIV particles to CD4(+) cells. *J Biol Chem.* 273:21988-97.

Carney, D.N., A.F. Gazdar, G. Bepler, J.G. Guccion, *et al.* 1985. Establishment and identification of small cell lung cancer cell lines having classic and variant features. *Cancer Res.* 45:2913-23.

Davis, K.A., Y. Lin, B. Abrams, and S.D. Jayasena. 1998. Staining of cell surface human CD4 with 2'-F-pyrimidine-containing RNA aptamers for flow cytometry. *Nucleic Acids Res.* 26:3915-24.

Derenzini, M. 2000. The AgNORs. *Micron.* 31:117-20.

Gey, G., W. Coffman, and M. Kubicek. 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. *Cancer Res.* 12:264.

Giaccone, G., J. Battey, A.F. Gazdar, H. Oie, *et al.* 1992. Neuromedin B is present in lung cancer cell lines. *Cancer Res.* 52:2732s-2736s.

Ginisty, H., H. Sicard, B. Roger, and P. Bouvet. 1999. Structure and functions of nucleolin. *J Cell Sci.* 112:761-72.

Goding, J.W. 1996. Monoclonal antibodies: Principles and Practice. Academic Press, San Diego. 492 pp.

Harlow, E., and D. Lane. 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. 726 pp.

Harlow, E., and D. Lane. 1999. Using antibodies: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Kim, C.S., B.B. Berkley, W.M. Abraham, and A. Wanner. 1982. A micro double capillary method for rheologic measurements of lower airway secretions. *Bull Eur Physiopathol Respir.* 18:915-27.

King, M., and D. Speert. US Patent 6,339,075. 2002. Use of dextran and other polysaccharides to improve mucus clearance.

Langer, P.R., A.A. Waldrop, and D.C. Ward. 1981. Enzymatic synthesis of biotin-labeled polynucleotides: novel nucleic acid affinity probes. *Proc Natl Acad Sci U S A.* 78:6633-7.

Lin, D.L., and C. Chang. 1996. p53 is a mediator for radiation-repressed human TR2 orphan receptor expression in MCF-7 cells, a new pathway from tumor suppressor to member of the steroid receptor superfamily. *J Biol Chem.* 271:14649-52.

Little, C.D., M.M. Nau, D.N. Carney, A.F. Gazdar, *et al.* 1983. Amplification and expression of the *c-myc* oncogene in human lung cancer cell lines. *Nature.* 306:194-6.

McNicol, A.M., and J.A. Richmond. 1998. Optimizing immunohistochemistry: antigen retrieval and signal amplification. *Histopathology.* 32:97-103.

Mickey, D.D., K.R. Stone, H. Wunderli, G.H. Mickey, *et al.* 1977. Heterotransplantation of a human prostatic adenocarcinoma cell line in nude mice. *Cancer Res.* 37:4049-58.

Miller, D., P. Bates, and J. Trent. WO 00/61597. 2000. Antiproliferative activity of G-rich oligonucleotides and method of using same to bind to nucleolin.

Naito, M., H. Hamada, and T. Tsuruo. 1988. ATP/Mg^{2+}-dependent binding of vincristine to the plasma membrane of multidrug-resistant K562 cells. *J Biol Chem.* 263:11887-91.

Orfao, A., and A. Ruiz-Arguelles. 1996. General concepts about cell sorting techniques. *Clin Biochem.* 29:5-9.

Robinson, J.M., and D.D. Vandre. 2001. Antigen retrieval in cells and tissues: enhancement with sodium dodecyl sulfate. *Histochem Cell Biol.* 116:119-30.

Rubin, B., and M. Newhouse. US Patent 5,925,334. 1999. Use of surface active agents to promote mucus clearance.

Shi, S.R., R.J. Cote, and C.R. Taylor. 1997. Antigen retrieval immunohistochemistry: past, present, and future. *J Histochem Cytochem.* 45:327-43.

Shi, S.R., R.J. Cote, and C.R. Taylor. 2001. Antigen retrieval techniques: current perspectives. *J Histochem Cytochem.* 49:931-7.

Sorokina, E.A., and J.G. Kleinman. 1999. Cloning and preliminary characterization of a calcium-binding protein closely related to nucleolin on the apical surface of inner medullary collecting duct cells. *J Biol Chem.* 274:27491-6.

Srivastava, M., and H.B. Pollard. 1999. Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. *Faseb J.* 13:1911-22.

Stone, K.R., D.D. Mickey, H. Wunderli, G.H. Mickey, *et al.* 1978. Isolation of a human prostate carcinoma cell line (DU 145). *Int J Cancer.* 21:274-81.

Takahashi, T., M.M. Nau, I. Chiba, M.J. Birrer, *et al.* 1989. p53: a frequent target for genetic abnormalities in lung cancer. *Science.* 246:491-4.

Tockman, M.S., J.L. Mulshine, S. Piantadosi, Y.S. Erozan, *et al.* 1997. Prospective detection of preclinical lung cancer: results from two studies of heterogeneous nuclear ribonucleoprotein A2/B1 overexpression. *Clin Cancer Res.* 3:2237-46.

Tuteja, R., and N. Tuteja. 1998. Nucleolin: a multifunctional major nucleolar phosphoprotein. *Crit Rev Biochem Mol Biol.* 33:407-36.

van de Loosdrecht, A.A., R.H. Beelen, G.J. Ossenkoppele, M.G. Broekhoven, *et al.* 1994. A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia. *J Immunol Methods*. 174:311-20.

Wysocki, L.J., and V.L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. *Proc Natl Acad Sci U S A*. 75:2844-8.

Xu, X., F. Hamhouyia, S.D. Thomas, T.J. Burke, *et al.* 2001. Inhibition of DNA Replication and Induction of S Phase Cell Cycle Arrest by G-rich Oligonucleotides. *J Biol Chem*. 276:43221-30.

Yao, G.Q., S. Corrias, and Y.C. Cheng. 1996a. Identification of two oligodeoxyribonucleotide binding proteins on plasma membranes of human cell lines. *Biochem Pharmacol*. 51:431-6.

Yao, G.Q., S. Corrias, and Y.C. Cheng. 1996b. Identification of two oligodeoxyribonucleotide binding proteins on plasma membranes of human cell lines. *Biochem Pharmacol*. 51:431-6.

CLAIMS

1. A method of determining a neoplastic state of a cell, comprising: detecting the presence of plasma membrane nucleolin in the cell.

2. The method of claim 1, wherein the cell is mammalian.

3. The method of claim 2, wherein the cell is monkey, dog, cat, rabbit, cow, pig, goat, guinea pig, mouse, rat or sheep.

4. The method of claim 3, wherein the cell is human.

5. The method of claim 1, wherein the cell is lysed.

6. The method of claim 1 wherein the detecting comprises detecting nucleolin-anti-nucleolin agent complex.

7. The method of claim 6, wherein the anti-nucleolin agent is a monoclonal antibody that specifically binds nucleolin.

8. The method of claim 7, wherein the antibody is p7-1A4 mouse monoclonal antibody, sc-8031, sc-9893, sc-9892, 4E2 or 3G4B2.

9. The method of claim 6, wherein the anti-nucleolin agent is a nucleolin-binding oligonucleotide.

10. The method of claim 9, wherein the oligonucleotide is a guanosine-rich oligonucleotide.

11. The method of claim 10, wherein the oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NOs:1-7; 9-17; 19-30 or 31.

12. The method of claim 6, wherein the complex is detected by detecting fluorescence, an enzyme, or radioactivity.

13. A method of determining a neoplastic state of a cell, comprising quantifying the amount of plasma membrane nucleolin in the cell.

14. The method of claim 13, wherein the cell is mammalian.

15. The method of claim 14, wherein the cell is monkey, dog, cat, rabbit, cow, pig, goat, guinea pig, mouse, rat or sheep.

16. The method of claim 15, wherein the cell is human.

17. The method of claim 13, wherein the cell is lysed.

18. The method of claim 13 wherein the quantifying comprises quantifying nucleolin-anti-nucleolin agent complex.

19. The method of claim 18, wherein the anti-nucleolin agent is a monoclonal antibody.

20. The method of claim 18, wherein the antibody is p7-1A4 mouse monoclonal antibody, sc-8031, sc-9893, sc-9892, 4E2 or 3G4B2.

21. The method of claim 18, wherein the anti-nucleolin agent is nucleolin-binding oligonucleotide.

22. The method of claim 21, wherein the oligonucleotide is a guanosine-rich oligonucleotide.

23. The method of claim 22, wherein the oligonucleotide comprises a sequence selected from the group consisting of SEQ ID NOs:1-7; 9-17; 19-30 or 31.

24. The method of claim 23, wherein the complex is quantified by quantifying fluorescence, an enzyme, or radioactivity.

25. A kit for determining a neoplastic state of a cell, comprising:
an anti-nucleolin agent and
a control sample.

26. The kit of claim 25, further comprising a second agent that detects a nucleolin-anti-nucleolin complex.

27. The kit of claim 26, wherein the anti-nucleolin agent is an anti-nucleolin antibody, and the second agent is a labeled secondary antibody.

28. The kit of claim 27, wherein the label is at least one enzyme, fluorophore, radioisotope or gold particle.

29. The kit of claim 28, wherein the anti-nucleolin agent comprises biotin, and the second agent comprises avidin or streptavidin.

30. The kit of claim 28, wherein the anti-nucleolin agent comprises digoxigenin, and the second agent comprises a labeled anti-digoxigenin antibody.

31 The kit of claim 25, further comprising at least one member selected from the group consisting of a fixative, a buffer, plasticware, serum, serum albumin, non-fat milk, membranes and instructions.

32. The kit of claim 25, wherein the anti-nucleolin agentt is labeled.

33. The kit of claim 32, wherein the label is at least one enzyme, fluorophore, radioisotope or gold particle.

34. A method of detecting small lung cell carcinoma in a subject, comprising:

collecting a sample from the subject containing lung cells; and

detecting the presence of nucleolin on the surface of the cells.

35. The method of claim 34, wherein the sample is sputum.

36. The method of claim 34, wherein the subject is human.

37. A method of diagnosing pre-malignant or malignant cells, comprising:

collecting a sample from a subject comprising lung cells;

sending the sample to a testing center;

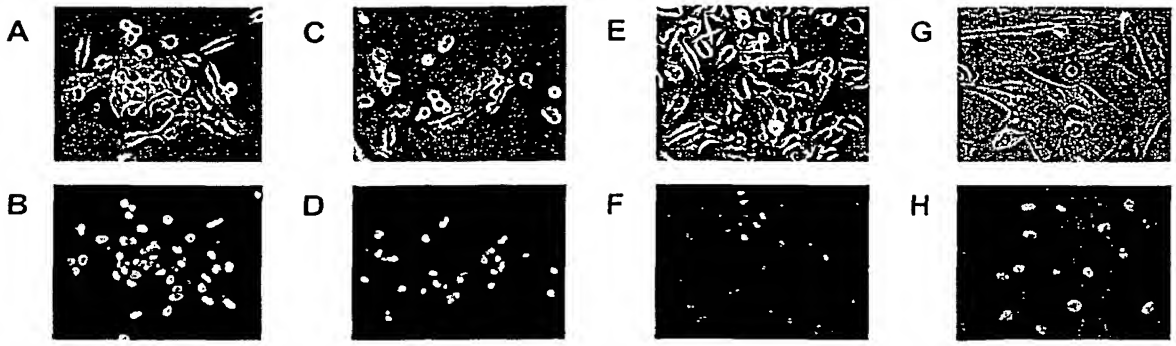
determining the neoplastic state of the cells by the method of claim 1.

38. The method of claim 38, wherein the subject is human.

39. The method of claim 39, wherein the sample is sputum.

40. The method of claim 39, wherein the diagnosing is diagnosing lung small cell carcinoma.

Figure 1



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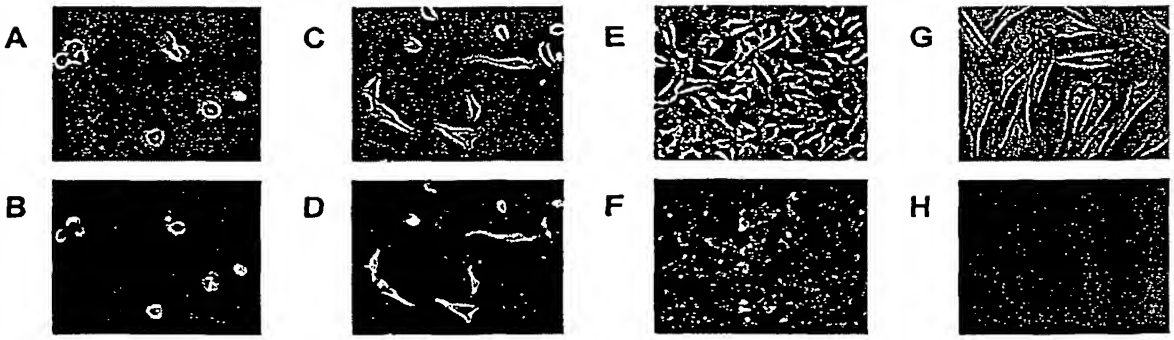

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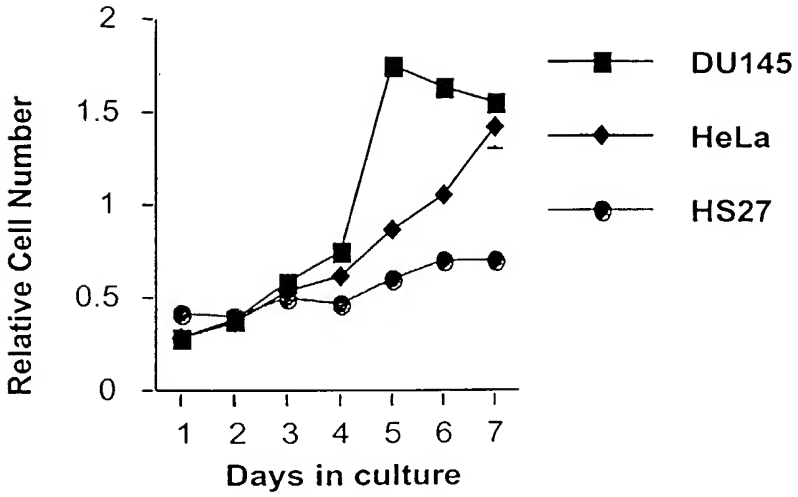
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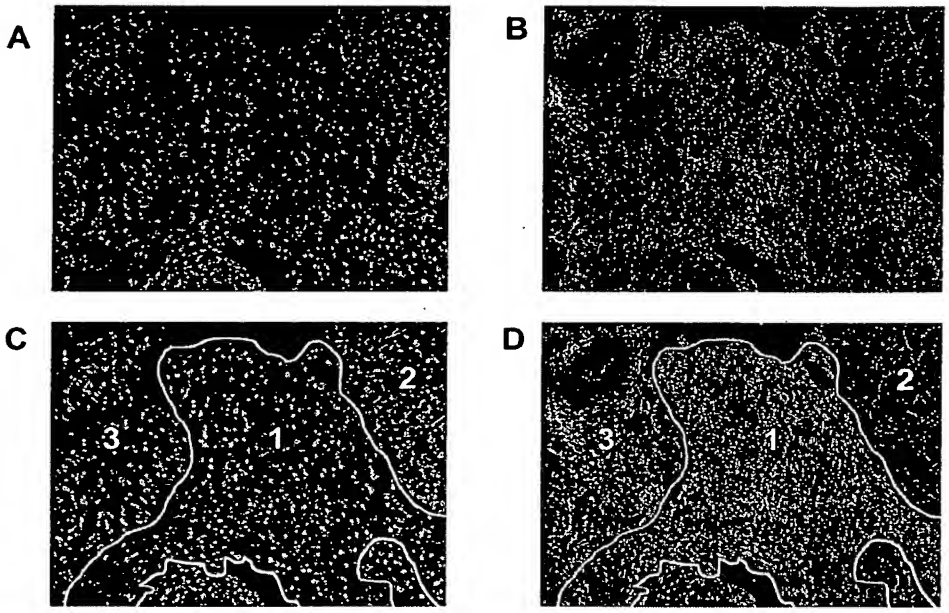

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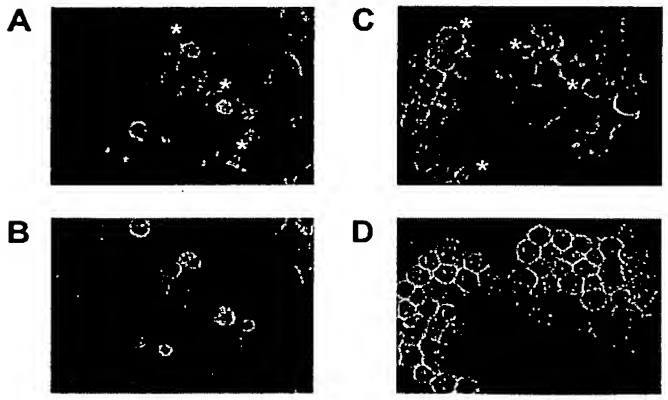
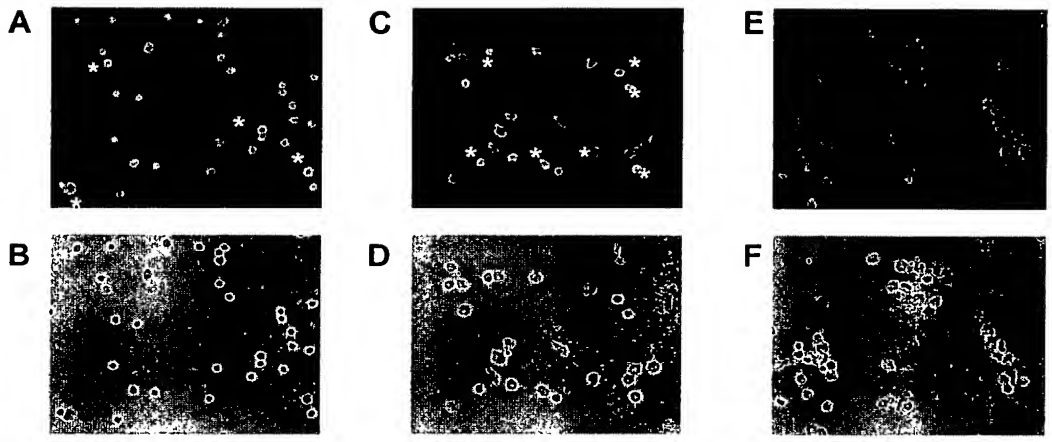


Figure 6



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